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Variability in Fecal Water Genotoxicity, Determined Using the Comet Assay, Is Independent of Endogenous *N*-Nitroso Compound Formation Attributed to Red Meat Consumption

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Red meat consumption causes a dose-dependent increase in fecal apparent total *N*-nitroso compounds (ATNC). The genotoxic effects of these ATNCs were investigated using two different Comet assay protocols to determine the genotoxicity of fecal water samples. Fecal water samples were obtained from two studies of a total of 21 individuals fed diets containing different amounts of red meat, protein, heme, and iron. The first protocol incubated the samples with HT-29 cells for 5 min at 4°C, whereas the second protocol used a longer exposure time of 30 min and a higher incubation temperature of 37°C. DNA strand breaks were quantified by the tail moment (DNA in the comet tail multiplied by the comet tail length). The results of the two Comet assay protocols were significantly correlated ($r = 0.35$, $P = 0.003$), however, only the second protocol resulted in detectable levels of DNA damage. Inter-individual effects were variable and there was no effect on fecal water genotoxicity by diet ($P > 0.20$), mean transit time ($P = 0.588$), or weight ($P = 0.705$). However, there was a highly significant effect of age ($P = 0.019$). There was no significant correlation between concentrations of ATNCs in fecal homogenates and fecal water genotoxicity ($r = 0.04$, $P = 0.74$). ATNC levels were lower in fecal water samples (272 μg/kg) compared to that of fecal homogenate samples (895 μg/kg) ($P < 0.0001$). Failure to find dietary effects on fecal water genotoxicity may therefore be attributed to individual variability and low levels of ATNCs in fecal water samples. Environ. Mol. Mutagen. 47:179–184, 2006.

Key words: meat; fecal water; comet assay; *N*-nitroso compounds; colon

INTRODUCTION

Up to 80% of colorectal cancers in Western populations are currently attributed to diet, which suggests that this cancer is a potentially preventable disease [Willet, 1995]. Diets high in red meat have been associated with an increased risk of colorectal cancer [Norat et al., 2002]. Previous studies have established that red meat, but not white meat, stimulates endogenous *N*-nitrosation in humans, and heme iron, specifically, can produce the same effect [Hughes et al., 2001; Bingham et al., 2002; Cross et al., 2003]; this may be pertinent for carcinogenesis in the large bowel since many classes of *N*-nitroso compounds (NOCs) have been identified, including nitrosamines, nitrosamides, and nitrosoguanidines, most of which are known carcinogens [Mirvish, 1995].

After consuming meat, the large intestine becomes rich in nitrogenous residues and nitrosating agents from chemical catalysis and bacterial dissimilatory nitrate metabolism; these processes lead to the production of amines and amides, which are in turn *N*-nitrosated in the presence of nitrosating agents such as nitric oxide (NO) [Mirvish, 1995; Tricker, 1997]. The microbiota in the large intestine reduce nitrate to nitrite, which can be converted to effec-

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tive nitrosating agents, and therefore, increase the potential for endogenous NOC production. The consequences of elevated endogenous NOC levels are not known.

The alkaline Comet assay is a sensitive technique that enables the detection of DNA strand breaks and alkali-labile sites in individual cells [Fairbairn et al., 1995; Holz et al., 1995]. Previous studies carried out using this technique showed that the known NOC carcinogen *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) was genotoxic in human colon cells [Pool-Zobel and Leucht, 1997]. In this present report, HT-29 cells were used to assess the genotoxicity of fecal water samples from individuals fed low red meat, high red meat, vegetarian, heme iron and iron diets, using both the standard protocol for the Comet assay [Venturi et al., 1997] and one in which conditions for DNA repair are minimised [Gill et al., 2004]. Results were compared with the previously published apparent total *N*-nitroso compound (ATNC) levels in fecal homogenates [Bingham et al., 2002; Cross et al., 2003]. We also compared ATNC levels in fecal water used for the Comet assay with ATNC levels in fecal homogenates from the same individuals.

MATERIALS AND METHODS

Study Design

The protocol for the dietary studies has been reported in full elsewhere [Bingham et al., 2002; Cross et al., 2003]. Briefly, the fecal water samples were collected from two randomized, controlled studies, with cross-over designs, that were conducted in a metabolic suite where diet could be carefully controlled and specimens collected. All diets were prepared by diet technicians as isocaloric and were weighed to the nearest gram. To keep nitrate intake constant, deionized water was provided for drinking and used in cooking, and low nitrate vegetables were used. All food was from the same batch, and stored for later use throughout the study to ensure minimal day-to-day variation. Six different diets, all of 15 days duration, were investigated. In the first study, 12 healthy male volunteers were fed a 60 g/day red meat diet, a 420 g/day red meat diet, and a vegetarian diet containing the same amount of protein as the 420 g/day red meat diet; meat was substituted in the vegetarian diet with egg, peanuts, low fat cheese, kidney beans, and green lentils. The 60 g/day red meat diet contained 65 g protein, and the 420 g/day red meat and vegetarian diets contained 143–150 g of protein. In the second study, nine healthy male volunteers were fed four different diets: a baseline diet consisting of a 60 g/day red meat diet (containing 9.9 mg/day iron), a 120 g/day red meat diet, the baseline diet supplemented with 7.8 mg/day heme iron (from liver pate and blood sausage) to match the iron content of the 420 g/day red meat diet (17.7 mg/day) and the baseline diet supplemented with a 300 mg/day (35 mg of ferrous iron) ferrous gluconate tablet (only six volunteers completed this diet). The diets contained less than 13 μg preformed NOC per day. Weight and age of the subjects was recorded and mean transit time measured using radio-opaque pellets, as described elsewhere [Cummings et al., 1976].

Fecal Homogenate Samples and ATNC Analysis

Fecal samples collected on days 10, 13, and 15 of each diet were immediately frozen on dry ice and further processed within 48 hr. Samples were diluted fourfold with ultra-pure deionized water, homogenized in a stomacher (Colworth 3500; Seward Medical, London, UK), and centrifuged at 3,400 × *g* for 10 min. Each supernatant was filtered through a 500-μm sieve and stored at −20°C before being analyzed for ATNCs by the release of NO following chemical denitrosation via thermal energy analysis [Pignatelli et al., 1987]. Results are presented as ATNC (μg/kg). Levels of ATNCs for the fecal homogenate samples have been previously published [Bingham et al., 2002; Cross et al., 2003]. In subjects (4, 6, and 7) from the second dietary study, ATNC concentration was measured in both fecal homogenates and their corresponding fecal water extracts. Three samples were selected from each of the three subjects for the 60 g/day red-meat diet and the 120 g/day red-meat diet ($n = 9$ in total/diet). Eight samples were analyzed from the heme iron supplement diet (three each from subjects 4 and 6, and two from subject 7).

Fecal Water Extraction and Genotoxicity

Fecal water samples were extracted from fecal samples collected on days 10, 13, and 15 of each diet, by homogenizing the sample for 2 min and then with centrifugation at 50,000 × *g* for 2 hr. The supernatant fecal water was aliquoted and stored at −80°C.

A human adeno-carcinoma cell line (HT-29) was obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, U.K.). The HT-29 cells were cultured for 7 days, harvested in 1% trypsin when they were still subconfluent, and resuspended in Dulbecco's Modified Eagles Medium supplemented with 100 U/l penicillin/streptomycin, 2 mmol/l glutamine, and 10% fetal calf bovine serum (all Gibco-Life Technologies, Paisley, Scotland). Cells were tested using Trypan blue for viability before use. Four hundred fifty microliters aliquots of cell suspension were incubated with 50 μl of each fecal water sample for 5 min at 4°C [Gill et al., 2004] in the first protocol, and for an extended incubation period and higher temperature (30 min at 37°C) [Venturi et al., 1997; Rieger et al., 1999] for the second protocol. The cells were then pelleted at 100 × *g* for 5 min and resuspended in 120 μl of 0.85% w/v low-melting-point agarose, and genotoxic potential was assessed using the COMET assay (single-cell gel electrophoresis) as outlined by Venturi et al. [1997]. Positive (25 μM hydrogen peroxide) and negative (0.9% saline) controls were included with each Comet assay experiment. Ileostomy fluid contains similar quantities of ATNC to that found in feces, and a quality control sample prepared from a large batch of ileostomy sample [Lunn et al., 2004] was included with each analytical run and scored to assess between-batch variation. All samples were blindly analyzed in triplicate. Quantification of the tail moment (i.e., the fractional amount of DNA in the comet tail multiplied by the length of the comet tail from the center of the head of the comet to the end of the tail) was used as the method of scoring. No difference in genotoxicity between samples from ileostomy subjects fed different diets was found when either tail moment or percentage DNA in the comet tail was used as the method of quantification [Lunn et al., 2004]. One hundred randomly selected cells were measured, and tail moments were recorded using KOMET 3.0 image analysis software (Kinetic Imaging, Liverpool, UK). The 75th percentile of the mean tail moment was calculated for each slide. Samples with a tail moment of <5 arbitrary units were classified as non-damaged, 5–17 as low, 17–32 as medium, and >32 as high levels of genotoxicity [Venturi et al., 1997].

Statistical Analysis

Statistical analysis was carried out using SPSS version 10.0 (SPSS, Chicago, IL). Sample sizes were determined according to differences in fecal NOC excretion as reported elsewhere [Cross et al., 2003]. Two-way ANOVA was used to determine the effects of protocol type and the differences between individual levels of fecal water genotoxicity. When an effect was apparent by two-way ANOVA, paired Students' t-tests were carried out. Spearmans' correlation coefficient was used to detect

TABLE I. The Effect of Different Incubation Conditions on the Mean Tail Moment Induced by Fecal Water Samples Using the Comet Assay

Study	Diet	Number of subjects	First protocol Mean (± standard deviation)	Second protocol mean (± SD)	*P* value for t-test	Mean fecal homogenate ATNC (μg/kg)
1	60 g/day red meat	12	2.86 ± 3.20	9.97 ± 9.19	0.007	295.51 ± 227.33
1	420 g/day red meat	12	2.38 ± 2.20	8.01 ± 7.43	0.023	1409.53 ± 930.53
1	Vegetarian	12	1.86 ± 1.37	7.18 ± 6.87	0.018	313.14 ± 210.36
2	60 g/day red meat	9	1.63 ± 0.77	10.19 ± 6.83	0.005	690.22 ± 700.18
2	120 g/day red meat	9	1.58 ± 1.06	10.06 ± 6.18	0.002	959.37 ± 890.53
2	Heme iron supplement	9	1.68 ± 0.94	8.21 ± 4.65	0.002	1536.01 ± 1560
2	Iron supplement	6	0.80 ± 0.38	9.98 ± 7.42	0.032	1203.98 ± 1619.99

relationships between variables. Probability results less than the 0.05 significance level were regarded as significant.

RESULTS

Quality Control Results

The negative control samples resulted in a mean (± standard deviation (SD)) tail moment of 0.86 ± 0.48 and the positive control samples resulted in a mean tail moment of 18.02 ± 4.69. The mean tail moment values for the quality control samples, from the pooled sample of ileostomy fluid, inserted into each of the 16 batches was 3.33 (SD = 0.60) and had a coefficient of variation of 18% and a range of 2.15–4.70.

Comet Assay Protocols and Fecal Water Genotoxicity in Relation to Diet

The results from the two methods (Table I) were significantly correlated ($r = 0.35$, $P = 0.003$), although they were significantly different ($P < 0.0001$). All mean values for the first protocol were 2.86 or below, and was categorized as non-damaged DNA; however, the mean values from the second protocol were categorized as low DNA damage, between 7.18 and 10.19. As no significant damage was induced by any fecal water samples using the first protocol conditions, no further results are discussed for the first protocol.

Table I shows mean tail moments for the different dietary studies, together with mean fecal homogenate ATNC levels as previously reported. There was no significant effect of dietary regime on the mean tail moment values, using two way ANOVA ($P = 0.269$ for the first dietary study ($n = 12$) and $P = 0.664$ for the second dietary study ($n = 6$–9)).

Inter-Individual Variation in Fecal Water Genotoxicity

Table I shows that standard deviations in individual values were high compared with that of the mean values. There were no significant correlations between weight ($r = 0.09$, $P = 0.45$) or mean transit time ($r = 0.02$, $P = 0.85$) and fecal water genotoxicity. However, there was a significant correlation between age and fecal water genotoxicity ($r = 0.39$, $P = 0.0011$), genotoxicity increasing with age. Individual 9 was the oldest participant of both dietary studies (see Figs. 1a and 1b). The youngest participants of the studies were individual 8 in the first study (Fig. 1a), and individual 14 in the second study (Fig. 1b).

Genotoxic Potential and ATNC Levels in Fecal Homogenate and Fecal Water Samples

There was no significant correlation between the concentration of ATNC in fecal homogenate samples and the genotoxicity of the corresponding fecal water samples ($r = 0.04$, $P = 0.74$) (Fig. 2). ATNC levels in the selected fecal water fractions were correlated with ATNC levels in the fecal homogenate samples ($r = 0.66$, $P = 0.0002$); however, ATNC levels in the fecal water fraction were significantly lower than those detected in the homogenate ($P < 0.0001$). The mean level of ATNC in the homogenate was 895 μg/kg, compared with a mean of 272 μg/kg in the fecal water fraction. Similar to the findings with ATNC in fecal homogenates, the fecal water ATNC did not correlate with mean tail moment ($r = 0.0043$, $P = 0.98$). Figure 3 shows differences between ATNC levels in the fecal water and the homogenate according to diet; all differences were significant ($P = 0.006$ for the heme iron supplement diet, $P = 0.012$ for the 120 g/day red-meat diet, and $P = 0.002$ for the 60 g/day red-meat diet).

DISCUSSION

This study investigated the potential of fecal ATNC to cause genotoxic damage in cultured human adeno-carcinoma cells, as assessed by the Comet assay. Two different Comet assay protocols were utilized to assess the optimum experimental conditions to investigate genotoxicity. Effects of diet, transit time, weight, and age of subjects on fecal water genotoxicity were considered. We also investigated what proportion of ATNCs is found in the fecal water extract compared with that of the fecal homogenate. HT-29 cells are of human colonic origin and have been widely assumed to be appropriate for assessing genotoxicity of

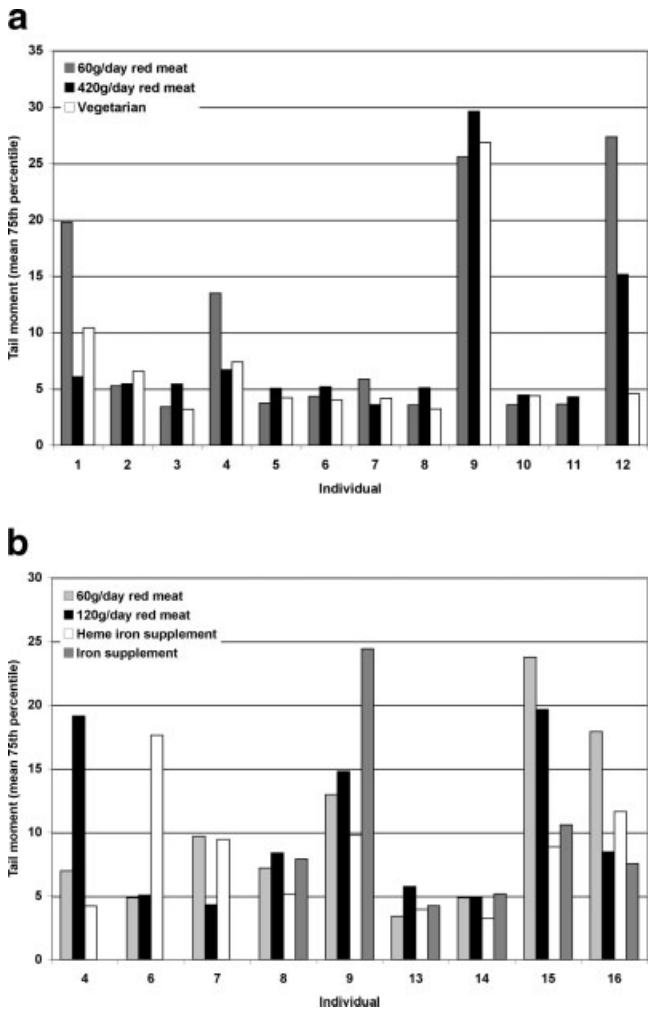

Fig. 1. Individual and dietary effects on fecal water genotoxicity using the second Comet assay protocol. Results from the first study are displayed in (**a**); results from the second study are displayed in (**b**).

human samples. Pool-Zobel and Leucht [1997] have previously shown that, using human colonic cells, the Comet assay is able to detect DNA damage arising from NOCs.

Although the two sets of data from the two different Comet assay protocols were correlated, the different incubation conditions had a highly significant effect on the resulting mean levels of genotoxicity. The second protocol mimicked the physiological conditions that colonic cells would naturally be subjected to, and therefore, seems to be more relevant to these studies. While the conditions used in the first protocol might have minimized the degree of DNA repair and therefore the degree of strand breaks; the overall level of DNA strand breaks were close to the normal background level of damage in these cells and classified as 'non-damaged' in all cases.

Previous studies of seven free-living volunteers indicated that diets high in meat and fat but low in dietary fiber increase the genotoxic potential of fecal water samples in HT-29 cells using the same Comet assay protocol used here (Protocol 2) [Rieger et al., 1999]. In contrast, we were unable to detect differences in genotoxicity attributable to diet, even in this comparatively large carefully controlled study. The lack of effect of diet on fecal water genotoxicity may have been due to a constant intake of fat throughout; however, we found that the inter-individual range in response was high, even on the same diet (Table I, and Figs. 1a, 1b, and 2). The lack of an effect of diet on fecal water genotoxicity shown here supports the findings of Hughes et al. [2002]; that is, there appears to be no association between fecal homogenate ATNC levels and fecal water genotoxicity.

Osswald et al. [2000] also found that fecal water genotoxicity varied in subjects receiving identical foods, and both a high inter- and intra-individual variability of fecal water genotoxicity was noted. A reason for this individual variation may be due to the individual variation in gastrointestinal microflora populations [Salminen et al., 1998]. *N*-nitrosation is thought to be catalyzed by bacteria colonizing the large intestine, since a study with germ-free rats revealed that for endogenous *N*-nitrosation to occur, an indigenous microbiota had to be present [Massey et al., 1988]. A number of facultative and anaerobic bacteria present in the gastrointestinal tract of humans are capable of nitrate- and nitrite-reducing activities via nitrate reductase [Calmels et al., 1985, 1988]. The activity of this enzyme has been positively correlated with nitrosating ability [Calmels et al., 1996], and has been shown to vary up to eightfold among individuals [Mallet et al., 1987], and could, therefore, explain individual variability in fecal ATNC levels.

Bingham et al. [submitted] are furthering investigations of the individual differences in fecal water genotoxicity by identifying the gut microflora from the fecal homogenate samples used in the present study. Burns and Rowland [2004] recently determined that certain strains of lactic acid bacteria were capable of counteracting the genotoxic potential of fecal water as a consequence of different end products of bacterial metabolism, such as short chain fatty acids. Furthermore, a number of lactic acid bacteria specifically inhibit NOC (MNNG) induced genotoxicity in rat colon cells [Pool-Zobel et al., 1996]. In the current study, we found an effect of increasing genotoxicity with age. Numbers of lactic acid bacteria (in particular bifidobacteria) decrease with age [Hopkins et al., 2001]. Studies by Lunn et al. [2004] suggest that the gut microflora may not be the main contributor to NOC production, as levels of ATNCs from ileum contents are similar to those found in the colon, yet bacterial microflora are present in lower numbers in the small intestine compared with that of the large intestine. This study also was unable to detect an effect of diet on genotoxicity using the same second protocol [Lunn et al., 2004].

The fecal water fraction of human feces is routinely used when studying the link between diet and colorectal

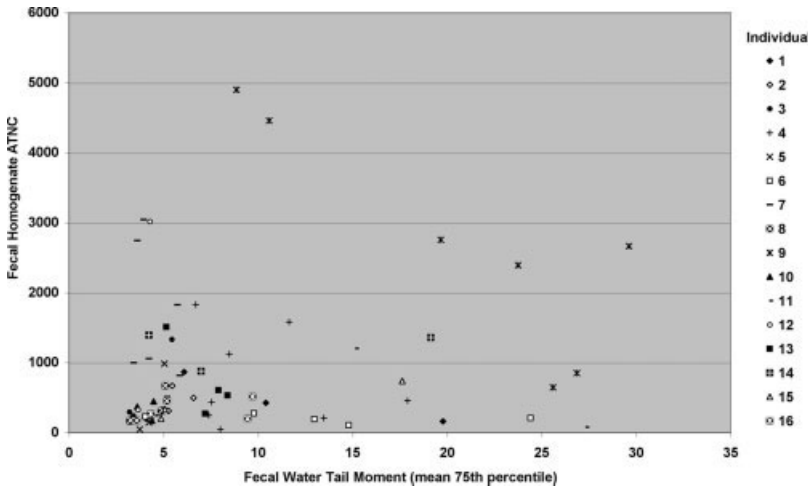

Fig. 2. Scattergram of the relationship between ATNC concentration in fecal homogenates and the genotoxicity of fecal water (second Comet assay protocol data).

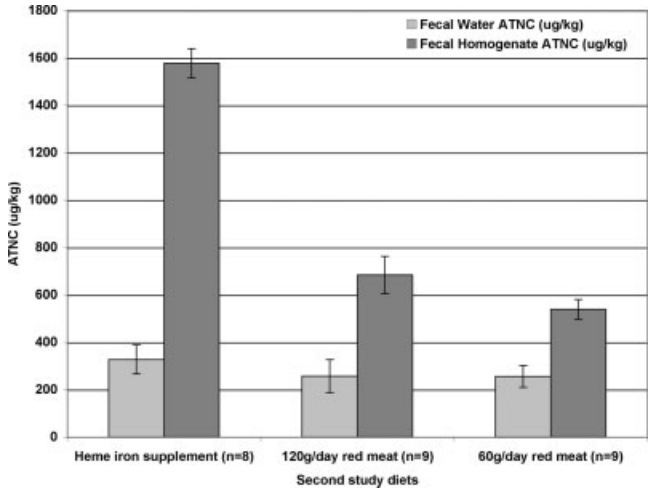

Fig. 3. Mean and standard errors for ATNC concentration in the fecal homogenate samples and their corresponding fecal water extracts. Twenty-six samples were analyzed in total from three subjects numbered 4, 6, and 7. Three samples were selected from each of three diets (60 g/day red meat, 120 g/day red meat, heme iron supplement) for individuals 4 and 6. Three samples from two diets (60 g/day red meat, 120 g/day red meat), and two samples from the heme iron supplement diet were selected for individual 7.

cancer using the Comet assay [Burns and Rowland, 2004; Klinder et al., 2004; Glei et al., 2005] because of the particulate nature of fecal homogenates, which interferes with the Comet, and other assays. Previous findings have shown that cytolytic agents are usually capable of exerting their effects only when in solution and that dietary changes may alter the composition of fecal water fractions [Rafter et al., 1987; Lapre and Van der Meer, 1992]. Moreover, the fecal water fraction is more efficient in altering the growth characteristics of colonocytes than components of the solid phase [Rafter and Branting, 1991; Lapre and Van der Meer, 1992]. Recently, Klinder et al. [2004] demonstrated a close association of fecal water genotoxicity and tumor risks in a rat study.

We have consistently found that red meat increases the level of endogenous ATNCs [Bingham et al., 2002; Cross et al., 2003]. We also have evidence that the NOCs investigated are direct acting nitrosated peptides since we recently identified the DNA adduct O^6-carboxymethyl guanine in exfoliated colonic cells isolated from human feces and in HT-29 cells. Furthermore, we have shown that the levels of this adduct increase in response to high red-meat diets [Lewin et al., submitted]. We would, therefore, have expected that red meat would increase fecal water genotoxicity, whereas we found no effect. However, we found that ATNC levels in fecal water samples are less than a third of the ATNC levels found in the corresponding fecal homogenate samples (Fig. 3); therefore, the use of the fecal water fraction may not be appropriate for some genotoxicity assessments in relation to diet.

To our knowledge, this is the first demonstration of a chemical difference between the fecal homogenate and fecal water. More precise determination of the particular NOC involved using mass spectrometry is complex, but is under investigation. In addition, DNA strand breaks are non-specific indicators of genotoxicity and the use of lesion-specific enzymes such as the enzyme 3-methyladenine DNA glycosylase II (AlkA), which incises DNA at 3-methyl adenines, would give more information in the Comet assay [Collins, 2004].

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